from RyR1 enhances the L-type Ca^{2+} current via the DHPR. RyR1 (5,037 residues) assembles as a homotetramer in which the C-termini form the ion conducting pore across the SR membrane, with the remainder of the protein forming a ''foot'' structure which spans between the SR and plasma membranes. In an accompanying abstract (Polster et al., this meeting) we showed that a construct (YFP-$RyR1_{1:4300}$) which lacks the C-terminal residues was diffusely distributed in dysgenic myotubes (DHPR null), but that in dyspedic myotubes (RyR1-null but containing DHPRs) it targeted junctionally and retrogradely enhanced L-type Ca^{2+} current. Here we have examined the oligomerization of YFP-$RyR1_{1:4300}$. Measurement of FRAP for YFP-$RyR1_{1:4300}$ in dysgenic myotubes yielded a diffusion coefficient of $2.17x10^{-8}$ cm^2/sec, which, based on previous measurements of protein diffusion in muscle, is compatible with YFP-$RyR1_{1:4300}$ existing as a tetramer. We also used SDS PAGE (4-15% gradient) and immunoblotting to compare YFP-$RyR1_{1:4300}$ (expressed in tsA-201 cells) and native RyR1. Without prior cross-linking, YFP-$RyR1_{1:4300}$ displayed a smaller apparent MW than wt RyR1; after glutaraldehyde cross-linking, both proteins migrated as single bands of much higher apparent MW, with YFP-$RyR1_{1:4300}$ again displaying a slightly greater mobility, consistent with it's being a tetramer.

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The Cytoplasmic Domain of the RyR1 Foot is Sufficient for DHPR (Cav1.1) Organization into Tetrads

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A RyR1 construct that lacks the channel forming C-terminal residues but includes the entire cytoplasmic foot region (YFP-$RyR1_{1:4300}$) forms a stable tetrameric structure (H. Bichraoui et al., abstract this meeting), colocalizes with DHPRs at SR/plasmalemma junctions and retrogradely enhances peak DHPR currents when expressed in dyspedic (RyR1 null) myotubes (A. Polster et al., abstract this meeting). We tested the interaction of the expressed $RyR1_{1:4300}$ with DHPRs in dyspedic myotubes by examining the DHPR disposition using freeze-fracture. Normally DHPRs are targeted to junctional sites in the absence of RyR, but their organization into tetrads and the arrangement of tetrads in ordered arrays is strictly dependent on their link to arrays of tetrameric RyRs. Thus the disposition of DHPRs in freeze-fracture images provides direct information both on DHPR/RyR interaction and on the arrangement of RyRs. In dyspedic cells DHPR clusters at peripheral couplings in a loose, completely random arrangement. Cells expressing full-length RyR1 show more tightly clustered DHPRs and the presence of complete (4 elements) and incomplete (2-3 elements) tetrads that are aligned in an orthogonal array. Cells expressing the truncated $RyR1_{1:4300}$ show small well identifiable DHPR foci with closely spaced particles. Some but not all foci show clear grouping of DHPRs into complete tetrads and/or tetrads composed of only three elements, but the tetrads are not aligned into a orthogonal array. We conclude that similar to intact RyR1, $RyR1_{1:4300}$ forms homo-tetramers which can link to DHPRs and organize them into tetrads, but that the cytoplasmic RyR1 foot differs from full-length RyR1 in that it does not form orthogonal arrays. The related positioning of $RyR1_{1:4300}$ and DHPRs is necessary for the retrograde interaction between the two.

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Two Regions are Involved in Ca^{2+}-Dependent Inactivation of Ryanodine Receptor Calcium Channels

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Skeletal (RyR1) and cardiac muscle (RyR2) isoforms of ryanodine receptor calcium channels are ~65 % homologous in amino acid sequences; however they differ in their regulation by endogenous molecules and proteins. Both RyR1 and RyR2 are inhibited by millimollar Ca^{2+}, but RyR2 affinity for inhibitory Ca^{2+} is ~10 times lower than RyR1. Previous studies demonstrated that C-terminus quarter of RyR has critical domain(s) for Ca^{2+} inactivation (Du and MacLennan (1999) *J. Biol. Chem.*274, 26120; Nakai et al. (1999) *FEBS Lett.*459, 154). We pursued these observations to obtain further insights into RyR regulation by Ca^{2+}. We constructed and expressed 8 RyR1/RyR2 chimeras in HEK293 cells and determined Ca^{2+} activation and inactivation affinities of these channels by [3H]ryanodine ligand binding method. We found that replacing two regions of RyR1 with corresponding RyR2 sequence reduced the affinity for Ca^{2+} inactivation. The first region (RyR2 amino acid 4020-4250) contains two EF hand Ca^{2+} binding motifs (EF1: 4036-4047, EF2: 4071-4082). Another chimera containing only EF2 of RyR2 (RyR2 4053-4250) has only a modest (not significant) change in Ca^{2+} inactivation. The results suggest that EF1 is a critical determinant for RyR inactivation by Ca^{2+}. Consistently, a chimera channel carrying only RyR2-EF1 (RyR2 3692-4052) showed significantly reduced Ca^{2+} inactivation, whereas a chimera carrying RyR2 3692-4019 (no EF hands) behaved essentially as RyR1. Second, preliminary studies indicate that substitution of the last ~450 amino acids of RyR1 with corresponding RyR2 (4521-4968) results in Ca^{2+} inactivation affinity between RyR1 and RyR2. Replacing both regions in RyR1 with RyR2 sequences (4020-4250 and 4521-4968) further reduced Ca^{2+} inactivation affinity, which suggests that in addition to EF1, C-terminal ~450 amino acids have a role in Ca^{2+}-dependent inactivation of RyRs. Supported by NIH, AHA and NSF.

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FRET Detection of CaM-RyR2 Binding Modulation by S100A1

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Using fluorescence resonance energy transfer (FRET), we are testing the hypothesis that S100A1 competes with calmodulin (CaM) for binding to cardiac ryanodine receptors (RyR2). In isolated pig cardiac sarcoplasmic reticulum (SR) vesicles, we targeted a donor-labeled FKBP (D-FKBP) to the RyR2 cytosolic headpiece. We then detected FRET as a decrease of donor fluorescence in the presence of CaM labeled with acceptor within the N- or C-lobe (denoted A_N-CaM and A_C-CaM, respectively), thus directly and specifically indexing CaM binding in the proximity of D-FKBP. FRET between D-FKBP and A-CaMs (100 nM) was completely inhibited by unlabeled WT-CaM, with $K_I \approx 100$ nM, indicating that WT-CaM and A-CaMs compete with similar affinities for the same RyR2 binding site. However, S100A1 (ranging from 0.1 to 30 μM) had no significant effect on FRET when cardiac SR was concurrently incubated with S100A1 and A_N-CaM. Upon sequentially incubating the SR with S100A1 first, then with A_N-CaM, we found partial inhibition of FRET, but with $K_I \approx 30$ μM S100A1. This effect is more pronounced in nM Ca^{2+} (*versus* μM Ca^{2+}). Intriguingly, FRET between D-FKBP and A_C-CaM was not significantly affected by S100A1. S100A1 lowered maximum FRET for A_N-CaM but did not significantly change its binding affinity. This suggests that S100A1 allosterically interacts with RyR-CaM binding, rather than directly competing for the same binding site as CaM. We are currently developing a complementary FRET approach, using acceptor-labeled S100A1, to specifically resolve the S100A1 binding to RyR. Ultimately, we aim to elucidate the interplay between S100A1 and CaM binding to RyR.

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Benzodiazepines and Benzothiazepines as Modulators of the Sarcoplasmic Reticulum Calcium ATP-ase and Ryanodine Receptors in Striated Muscle

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We have reported that CGP-37157, a benzothiazepine (BZT) derivative of clonazepam utilized as a blocker of the mitochondrial Na^+/Ca^{2+} exchanger, also activates ryanodine receptors (RyRs) and inhibits the sarcoplasmic reticulum (SR) Ca^{2+}-stimulated ATPase (SERCA). We extended the studies to other BZT (e.g., clonazepan, diltiazem) as well as to benzodiazepines (BZD; e.g. diazepan, lorazepan). We aimed to determine if these drug classes have as a common trait the ability to modulate RyRs and/or SERCA. The effects of BZD and BZT on RyRs activity were tested in SR microsomes with a Ca^{2+} leak assay as well as after reconstituting RyRs into lipid bilayers. The agents tested had variable potency to increase RyR-mediated Ca^{2+} leak from skeletal SR microsomes. As an example, while diazepam significantly increased RyR activity, most of the others (clonazepam, lorazepam) had minor effects at high doses. In contrast, diltiazem produced moderate inhibition. Planar bilayer studies confirmed the leak observations with both cardiac and skeletal RyR. In the presence of ruthenium red, most agents decreased the rate of loading which indicates inhibitory effects on SERCA activity. The effects of BZT and BZD on loading correlated with a decrease in ATPase activity of SERCA-enriched skeletal SR fractions. In summary, most BZT and BZD utilized in therapeutics or as pharmacological tools have an inhibitory action on SERCA. In contrast, they show a variety of effects on RyRs ranging from inhibition to activation. Hence, the pharmacological action of BZT and BZD on cellular Ca^{2+} homeostasis reported in the literature of cardiac and skeletal muscle as well as other non-muscle systems may require taking into consideration the contributions of all drug-sensitive intracellular Ca^{2+} transporters. (Supported by NIH-GM078665 and AHA-MWA 12180038).